XP-002107028 J. Med. Chem. 1997, 40, 3077-3084

Inhibition of Melanoma Cell Binding to Type IV Collagen by Analogs of Cell Adhesion Regulator

Janelle L. Lauer,[†,‡] Leo T. Furcht,[†,‡] and Gregg B. Fields*[,†,‡,§,▽]

Departments of Laboratory Medicine and Pathology and of Biochemistry and The Biomedical Engineering Center, University of Minnesota, Minneapolis, Minnesota 55455

Received March 27, 1997[⊗]

Integrin-mediated tumor cell adhesion to type IV collagen is believed to play a role in the invasion of basement membrane proteins and the subsequent metastatic process. The cellular protein CAR (cell adhesion regulator) has been proposed to influence integrin-mediated binding to extracellular matrix proteins, including basement membrane (type IV) collagen. Three analogs of the $CAR_{138-142}$ have been tested for activity. The first contains the 138−142 sequence ($CAR_{138-142}$, Val-Glu-Ile-Leu-Tyr-NH_2), the second contains the 138−142 sequence with a phosphorylated Tyr [$pCAR_{138-142}$, Val-Glu-Ile-Leu-Tyr(PO_3H_2)-NH_2], and the third contains the reversed 138−142 sequence ($rCAR_{138-142}$, Tyr-Leu-Ile-Glu-Val-NH_2). When added extracellularly, none of the analogs had a significant affect on cell adhesion to type IV collagen. Using a novel reversible cell permeabilization method, we found that intracellular incorporation of both $CAR_{138-142}$ and $pCAR_{138-142}$ resulted in inhibition of cell adhesion in a dose-dependent fashion. The IC_{50} values were ~90 and ~10 μM for $CAR_{138-142}$ and $pCAR_{138-142}$, respectively. Intracellular incorporation of the $rCAR_{138-142}$ peptide had no affect on cell adhesion. Fluorescence microscopy of a fluorescein-labeled $CAR_{138-142}$ peptide revealed that the reversible permeabilization procedure resulted in the peptides crossing the cell membrane. Affinity chromatography of melanoma cell lysates with $pCAR_{138-142}$ or $rCAR_{138-142}$ attached to a solid support of magnetic beads suggested that one protein was bound uniquely by $pCAR_{138-142}$. Immunoprecipitation analysis identified vinculin, a protein associated with the actin cytoskeleton, as the protein specifically bound by $pCAR_{138-142}$. Immunoprecipitation with $pp125^{FAK}$- or β1-integrin-derived mAbs gave negative results. Our study suggests that a possible therapeutic approach for inhibition of melanoma cell adhesion adhesion to extracellular matrix proteins is the use of CAR peptide analogs intracellularly.

Introduction

Integrin family $\alpha\beta$ heterodimers mediate cell−matrix and cell−cell adhesion. These integrin receptors are regulated by a complex signaling framework that can effect changes in integrin−ligand affinity, receptor clustering, and integrin−cytoskeletal interactions.[1] Much of the integrin-related research to date has focused on the affects of extracellular ligands on receptor function and downstream signaling events. Modulation of receptor activity can also be affected by "inside-out" signaling. This process involves signals being transduced via the integrin cytoplasmic tail back to the extracellular domain to alter ligand affinity.[2] The interruption of this "inside-out" signaling pathway using compounds incorporated into the intracellular environment could be a valuable tool in regulating integrin function. Cytosolic ligands could be used in this manner to interfere with many cellular processes, including tumor cell adhesion to and invasion through extracellular matrix (ECM).

One focus of cancer research has been the attempted correlation of tumor cell integrin expression and metastatic potential. While it is difficult to find consistent trends across various tumors, increased expression of the $\alpha_2\beta_1$-integrin has been shown to be directly correlated to metastatic potential for human osteosarcoma cells[3-5] and human melanoma cells.[6,7] Amongst its various activities, the $\alpha_2\beta_1$-integrin binds with high affinity to the triple-helical domains on collagen,[8-10] mediates melanocyte and melanoma cell adhesion and motility on type IV collagen,[11-15] and initiates signal transduction pathways leading to the induction of matrix metalloproteinase-1.[16,17] Human melanoma cell adhesion to type IV collagen can be >70% inhibited by anti-α_2- or anti-β_1-integrin subunit monoclonal antibodies (mAbs).[15] Thus, the $\alpha_2\beta_1$-integrin appears to be one of the primary receptors for melanoma cell adhesion to type IV collagen, with the $\alpha_1\beta_1$-integrin playing a more minor role in the adhesion of tumor cells to type IV collagen.[8]

Intracellular targeting of receptors such as the $\alpha_2\beta_1$-integrin could inhibit tumor cell adhesion, spreading, and/or invasion. Pullman and Bodmer[18] identified a gene product that participated in the regulation of integrin mediated binding to ECM proteins including collagen and laminin. Transfection of cells with a cDNA clone resulted in increased adhesion to collagen without affecting the expression of integrin subunits.[18,19] The enhanced adhesion could be inhibited with anti-α_2- or -β_1-, but not -α_3-, or -β_2-, integrin subunit mAbs. The proposed protein product of this gene was named cell adhesion regulator (CAR). Polymorphism at the CAR locus has been observed.[20] The CAR gene encodes for a 142-amino acid protein, which contains a putative myristoylation site at the *N*-terminus[18] and thus is

* Address correspondence to this author at: Department of Laboratory Medicine and Pathology, Box 609, 420 Delaware St. S.E., University of Minnesota, Minneapolis, MN 55465. Phone: 612-626-2446. Fax: 612-625-1121.

† Department of Laboratory Medicine and Pathology.

‡ The Biomedical Engineering Center.

§ Department of Biochemistry.

▽ Recipient of an NIH Research Career Development Award.

⊗ Abstract published in *Advance ACS Abstracts*, August 15, 1997.

S0022-2623(97)00206-9 CCC: $14.00

anticipated to be membrane bound. The last residue of CAR is proposed to be a Tyr phosphorylation site. When Tyr_{142} is converted to a stop codon, cell adhesion levels are reduced to pretransfection levels. Thus, CAR may control integrin binding via modulation of signal transduction and/or direct interaction with integrins.

We envision that intracellular incorporation of CAR analogs may represent a therapeutic approach for modulating tumor cell integrin function. A peptide-based strategy has been used in a number of studies to interfere with intracellular processes such as agonist-induced nuclear translocation,[21] protein–protein interactions,[22] and receptor-signaling molecule complex formation.[23] Each study used different methods to introduce the peptide into the cytosol, including the attachment of hydrophobic leader sequences to the target peptides[21] or enzymatic[22] or detergent-based[23] permeabilization procedures. In the present study, we have examined the effects of peptide models of $CAR_{138-142}$ on human melanoma cell adhesion to type IV collagen. Both extracellular and intracellular peptide interactions were tested. For the latter case, a reversible permeabilization method was modified so that it would be compatible with cell adhesion assays. Both the phosphorylated and nonphosphorylated sequences have been examined to determine if Tyr_{142} phosphorylation is required for CAR activity. To evaluate the specificity of the CAR sequence, a reversed sequence analog was also studied as a potential negative control. Another analog of the $CAR_{138-142}$ sequence was synthesized with a fluorescent tag and used in conjuction with fluorescence microscopy to determine if the peptides crossed the cell membrane. Finally, isolation and identification of cellular proteins that bind to the $CAR_{138-142}$ sequence were attempted by peptide affinity purification and immunoprecipitation methods.

Results

To study the influence of CAR model peptides on melanoma cell adhesion to type IV collagen, a reversible cell permeabilization procedure was needed that would not interfere with adhesion receptor function. The TransPort kit utilizes a water soluble lipid derivative for permeabilization. The derivative is then absorbed with a protein solution, terminating the process. After reversal of permeabilization, cells remain viable as demonstrated by [^{3}H]thymidine incorporation into DNA.[24] By measuring cellular incorporation of trypan blue, we found that >80% permeabilization could be produced with a 10 min incubation of the lipid derivative. Once the lipid derivative was quenched using the protein solution, the cells were plated and placed at 37 °C. After 30 min, ~50% of the trypan blue was retained, indicating that ~50% of cell membranes had resealed. After 60 min, the percentage of cells with intact membranes was 75–90%. The cells began to attach and spread after 90 min.

In an effort to modify the standard adhesion assay so that the effects of permeabilization could be minimized, adhesion levels were determined over a time course of recovery and adhesion times. The goal was to determine the length of time the permeabilized cells would need to recover before comparable levels of adhesion could be reached for permeabilized and nonpermeabilized cells. Melanoma cell adhesion to type IV collagen was examined. The cells were permeabilized for 10 min, followed by membrane "recovery" for 60, 90, or 120 min, and then cell adhesion for 60, 90, or 120 min (Table 1). The recovery time producing the greatest adhesion was either 60 or 90 min. A duration of 120 min for adhesion was found to be optimal. The treatment of 60 min for recovery followed by 120 min for adhesion produced adhesion levels in the permeabilized cells that were comparable to nonpermeabilized cells (106.3 ± 1.0% compared to 100.0 ± 0.7% of control). These conditions were primarily utilized throughout the study.[25,26] The longer recovery time (120 min) produced decreased levels of adhesion possibly due to (i) cellular production of ECM proteins, which could inhibit adhesion directly by competing for integrin binding sites, or (ii) induction of cellular aggregation. Decreases in cell adhesion with increasing time have been documented previously for nonpermeabilized cells.[27]

Table 1. Time Course To Determine Appropriate Postpermeabilization Adhesion Conditions

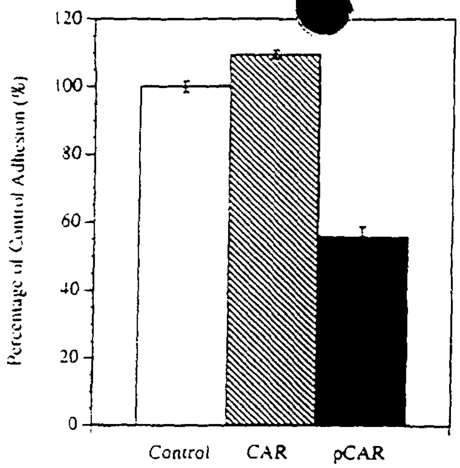
membrane recovery time (min)	adhesion time (min)	percentage of control adhesion (%)
control (no permeabilization)	60	100.0 ± 1.3
control (no permeabilization)	120	100.0 ± 0.7
60	60	42.9 ± 1.2
60	90	74.2 ± 2.6
60	120	106.3 ± 1.0
90	60	50.6 ± 0.4
90	90	84.3 ± 0.2
90	120	103.6 ± 1.6
120	60	28.1 ± 0.6
120	90	56.9 ± 0.5
120	120	30.0 ± 0.8

Since adhesion to type IV collagen is mediated by the β_1-integrin family, the postpermeabilization integrity of the β_1-integrin subunit is critical for our assay system. Integrin integrity was tested by comparing the ability of an anti-β_1-integrin mAb to inhibit permeabilized and nonpermeabilized cell adhesion to type IV collagen. The level of inhibition of adhesion to type IV collagen by 5 μg of the anti-β_1-integrin mAb for permeabilized and nonpermeabilized cells was 35 ± 1% and 55 ± 6%, respectively (data not shown), indicating that permeabilization did not significantly affect the ability of the β_1-integrin to mediate melanoma cell binding to type IV collagen. The difference in these values is due to variability of mAb activity.[15]

Initial experiments established the efficiency of both the $CAR_{138-142}$ and $pCAR_{138-142}$ peptides for inhibiting melanoma cell binding to type IV collagen. When cells were permeabilized, 10 μM $pCAR_{138-142}$ insertion resulted in 55% of control adhesion compared to 109% of control adhesion produced by $CAR_{138-142}$ insertion (Figure 1). Without permeabilization, neither 10 μM $CAR_{138-142}$ nor 10 μM $pCAR_{138-142}$ inhibited cell adhesion to type IV collagen (Figure 2), thus establishing the specificity of intracellular incorporation of peptide for activity.

The concentration dependence for inhibition of cell adhesion was then examined. When cells were not permeabilized, neither $CAR_{138-142}$ nor $pCAR_{138-142}$ significantly inhibited cell adhesion over a peptide concentration range of 1–100 μM (Figure 2). For permeabilized cells, both $CAR_{138-142}$ and $pCAR_{138-142}$ gave dose-dependent inhibition of melanoma cell adhesion to type IV collagen (Figure 3). The IC_{50} values were ~90

Journal of Medicinal Chemistry, 1997, Vol. 40, No. 19 3079



igure 1. Melanoma cell adhesion to type IV collagen in the resence of no peptide (open bar), 10 μM CAR$_{138-142}$ (striped ar), and 10 μM pCAR$_{138-142}$ (solid bar). Cells were permeabiized for 10 min, allowed to recover for 60 min, and allowed to dhere for 120 min. All assays were repeated a minimum of hree times.

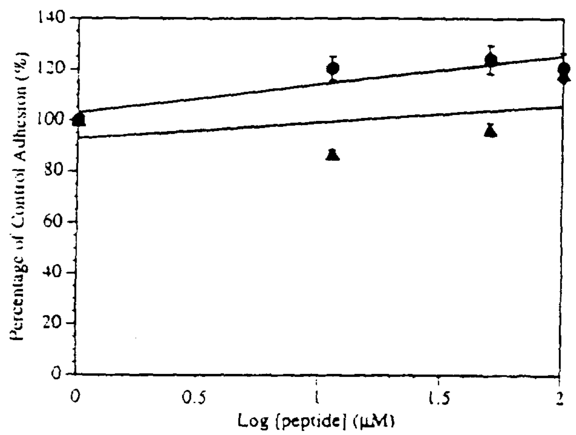


Figure 2. Inhibition of cell adhesion to type IV collagen by CAR$_{138-142}$ and pCAR$_{138-142}$ as a function of peptide concentration. Cells were treated with CAR$_{138-142}$ (▲) or pCAR$_{138-142}$ (●) and allowed to adhere for 120 min. All assays were repeated a minimum of three times.

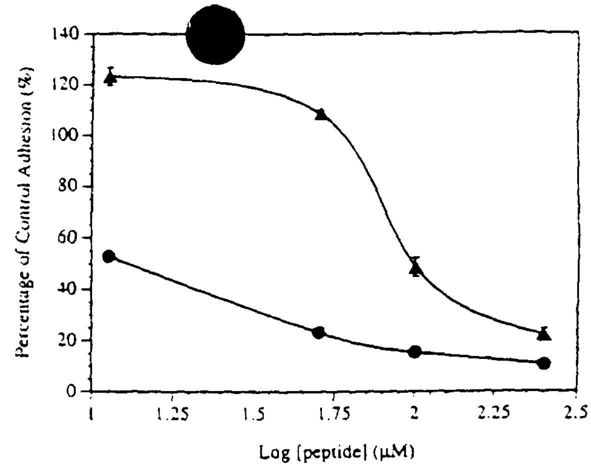


Figure 3. Inhibition of cell adhesion to type IV collagen by CAR$_{138-142}$ and pCAR$_{138-142}$ as a function of peptide concentration. Cells were treated with CAR$_{138-142}$ (▲) or pCAR$_{138-142}$ ( ), permeabilized for 10 min, allowed to recover for 60 min, and allowed to adhere for 120 min. All assays were repeated a minimum of three times.

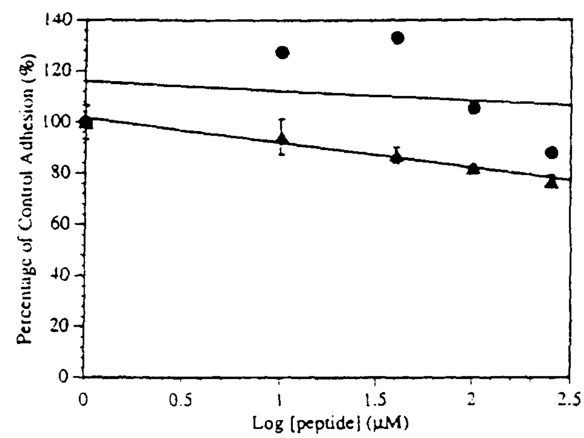


Figure 4. Inhibition of cell adhesion to type IV collagen by rCAR$_{138-142}$ as a function of peptide concentration. Cells were treated with rCAR$_{138-142}$ and either not permeabilized (▲) or permeabilized (●) for 10 min, allowed to recover for 120 min, and allowed to adhere for 120 min. All assays were repeated a minimum of three times.

and ~10 μM for CAR$_{138-142}$ and pCAR$_{138-142}$, respectively. No additional inhibition of cell adhesion was seen when the cells were treated with both 100 μM pCAR$_{138-142}$ and 5 μg of either an anti-α_2- or -β_1-integrin subunit mAb (data not shown). This indicates that the effects of pCAR$_{138-142}$ are integrin related. No significant inhibition was seen with increasing concentrations of the reverse sequence peptide rCAR$_{138-142}$ (1–100 μM) for either permeabilized or nonpermeabilized cells (Figure 4), thus establishing (i) the specificity of the CAR 138–142 sequence and (ii) that the permeabilization procedure itself did not affect cell adhesion.

To determine if the permeabilization method was indeed effective at allowing CAR analogs to penetrate the cell, we prepared a fluorescent CAR peptide (fCAR$_{138-142}$). Nonpermeabilized and permeabilized cells were incubated with 100 μM fCAR$_{138-142}$ at 37 °C for 10 min and then transferred to glass slides. Fluorescent microscopic images of the nonpermeabilized cells indicated that, in general, fluorescence appeared around the outside of the cell and faded rapidly over time (Figure 5, top). In contrast, fluorescent microscopic images of the permeabilized cells indicated that the fCAR$_{138-142}$ peptide crossed the cell membrane, as the cytoplasm appeared fluorescent (Figure 5, bottom).

CAR is predicted to bind directly to cellular proteins, thus modulating adhesion.[18] Affinity isolation and immunoprecipitation methodologies were used to identify any proteins bound to the pCAR$_{138-142}$ sequence. Since the pCAR$_{138-142}$ peptide was more effective than either CAR$_{138-142}$ or rCAR$_{138-142}$ at inhibiting cell adhesion, pCAR$_{138-142}$ was used for affinity isolation of cellular proteins. Tosyl-activated magnetic beads were used as a solid support, allowing for specific and reproducible peptide binding via the N-terminal primary amine. Cell lysates were first precleared twice using tris-blocked magnetic beads to remove any proteins binding nonspecifically to the solid support. The precleared lysates were then added to the peptide-coated beads. SDS–PAGE analysis of the elution products indicated four proteins bound to pCAR$_{138-142}$ (Figure 6A). The approximate molecular masses were 120–140, 60–65, 45, and 28–32 kDa. These four proteins were seen prominently in the bound fraction, not in the

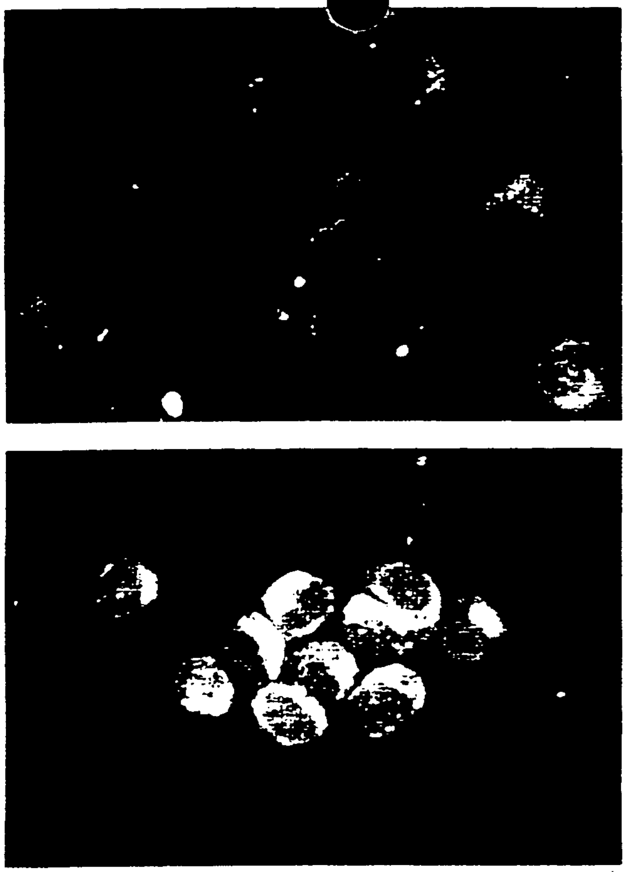


Figure 5. Fluorescent microscopic images of either nonpermeabilized (top) or permeabilized (bottom) melanoma cells treated with 100 μM fCAR$_{138-142}$ and 0.003% trypan blue. Fluoresence was monitored at $\lambda_{excitation}$ = 450 nm and $\lambda_{emission}$ = 535 nm.

precleared or wash fractions (Figure 6A). For comparison, affinity isolation of cell proteins was repeated using rCAR$_{138-142}$ bound to magnetic beads. SDS-PAGE analysis of the elution products indicated several proteins bound to rCAR$_{138-142}$ (Figure 6B). Among these proteins were three of approximate molecular masses 60–65, 45, and 28–32 kDa (Figure 6B), thus the 120–140 kDa protein was the only one specifically bound by pCAR$_{138-142}$. The pCAR$_{138-142}$-bound protein fraction was further analyzed by immunoprecipitation with mAbs against intracellular or membrane-spanning proteins of MW ~120–140 kDa. Vinculin was immunoprecipitated from the protein fraction, whereas pp125^{FAK} and the β_1-integrin subunit were not (Figure 7). Two additional bands, presumably degradation products of vinculin, were also detected in the immunoprecipitate as seen previously in vinculin immunoprecipitations.[28]

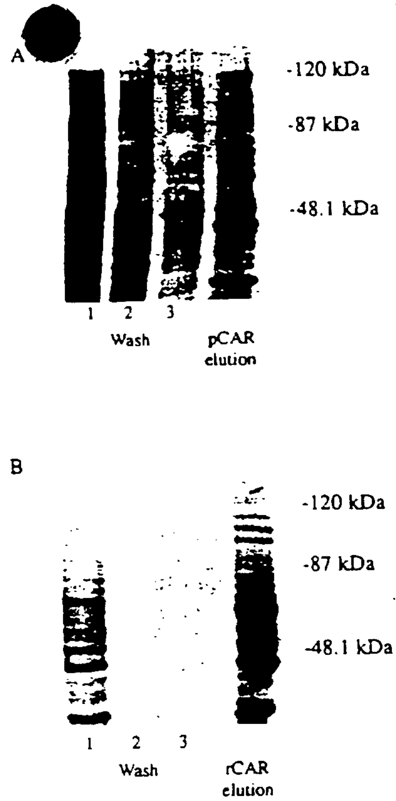


Figure 6. pCAR$_{138-142}$ and rCAR$_{138-142}$ affinity isolation of melanoma cell proteins. (A) Biotinylation/SDS–PAGE analysis of proteins isolated from melanoma cell lysates incubated with pCAR$_{138-142}$-coated magnetic beads. Lanes 1–3 are sequential PBS washes of the pCAR$_{138-142}$ beads after incubation with the cell lysates, and lane 4 shows the proteins eluted from the pCAR$_{138-142}$ beads with Laemmli buffer. (B) Biotinylation/SDS–PAGE analysis of proteins isolated from melanoma cell lysates incubated with rCAR$_{138-142}$-coated magnetic beads. Lanes 1–3 are sequential PBS washes of the rCAR$_{138-142}$ beads after incubation with the cell lysates, and lane 4 shows the proteins eluted from the rCAR$_{138-142}$ beads.

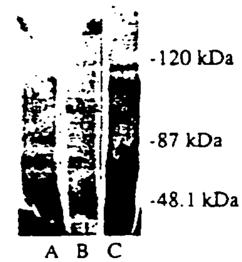


Figure 7. Immunoprecipitation analysis of proteins eluted from pCAR$_{138-142}$ with mAbs against (A) pp125^{FAK}, (B) β_1-integrin subunit, and (C) vinculin.

Discussion

The metastatic process involves tumor cell interaction with and invasion through the basement membrane, and thus the regulation of tumor cell integrins represents an important early step in metastasis. Since CAR appears to regulate cell adhesion to types I and IV collagen and laminin,[18,19] CAR may function by interaction with multiple integrins. We had previously demonstrated that human melanoma cell adhesion to basement membrane (type IV) collagen could be inhibited >70% by blocking the $\alpha_2\beta_1$-integrin.[15] Since the melanoma cell/type IV collagen adhesion system is a simple one, requiring primarily a single receptor for activity, one approach to modulating melanoma metastasis would be to inhibit the $\alpha_2\beta_1$-integrin. The intracellular incorporation of CAR peptide analogs could alter "inside-out" signal transduction, thus modulating $\alpha_2\beta_1$-integrin function so as to inhibit integrin-mediated binding to type IV collagen.

In order to quantitate the effects of CAR analogs, a reversible cell permeabilization procedure was required. We utilized a water soluble lipid derivative, which offers the advantages of being relatively mild and allowing 2–3 kDa peptides to be incorporated into the cell without affecting cell viability or receptor function

subsequent to permeabilization. Peptide insertion into cells has also been accomplished by tenolysin permeabilization,[22] but reversibility based on enzyme permeabilization is not possible. In order to test receptor-mediated activities such as adhesion, the reversibility of permeabilization is necessary. Treatment with detergents such as saponin permits peptides to enter the cell, as well as also large proteins such as antibodies.[23] Again, detergent preparation renders the cells irreversibly permeabilized. Endothelial cells can reestablish functional integrity following permeabilization using glass beads,[29] but the glass bead method produces rather large holes in the cell membrane (i.e., dextran of MW 152 kDa was incorporated into cells treated in this manner). The effects of this procedure on receptor integrity have not been studied. Electroporation has been used to insert peptides into cells,[30] but is also nonspecific, allowing for large protein (i.e., antibody) incorporation into cells.[31] Attachment of signal sequences to peptides can allow for transport across cell membranes,[21] but the extremely hydrophobic nature of such signal sequences limits this approach to a certain class of peptides, dependent on charge distribution. It is also possible that the addition of these signal sequences can affect the overall function of the target molecule. Most signal sequences are on the order of 15–20 amino acids in length. Once attached to a peptide the length of $CAR_{138-142}$, the signal sequence could interfere with the CAR peptide function within the cell. Alternatively, signal sequences could target the desired peptide to a specific cell type, where intracellular enzymes cleave the signal sequence leaving the peptide in the cytosol.[32] To date, this procedure does not have wide-ranging utility but is interesting conceptually and warrants further study.

CAR analogs were found to function as inhibitors of melanoma cell adhesion to type IV collagen when incorporated intracellularly. The *C*-terminal region of CAR, spanning residues 138–142, appears to influence integrin binding to type IV collagen. The specific sequence of $CAR_{138-142}$ is responsible for this behavior, as the reverse sequence has no activity. Affinity chromatography experiments were used to evaluate interactions of the $CAR_{138-142}$ sequence with cellular proteins. Cells were lysed, and the lysates were precleared twice with magnetic beads to remove proteins that bound nonspecifically to the solid support. Cell lysates were then reacted with $pCAR_{138-142}$-coated magnetic beads. The beads were washed extensively to remove unbound protein and then eluted with EDTA and guanidine hydrochloride. Simultaneously, cell lysates were reacted with $rCAR_{138-142}$-coated magnetic beads and eluted in similar fashion. One cellular protein bound uniquely to $pCAR_{138-14}$ compared with $rCAR_{138-142}$, indicating that a specific, direct interaction occurs between $pCAR_{138-142}$ and a cellular protein. The apparent molecular mass of this protein (120–140 kDa) suggested an integrin subunit, such as β_1 (120 kDa),[33] intracellular kinases, such as $p125^{FAK}$ (125 kDa) or $p130^{Cas}$ (130 kDa),[34,35] or cytoskeletal proteins, such as vinculin (117 kDa).[36] Immunoprecipitation analysis identified the isolated protein as vinculin, a protein implicated in modulating cell adhesion[37] and motility.[38] Thus, $pCAR_{138-142}$ binds specifically to vinculin, as evidenced by (i) binding of a 120–140 kDa protein to $pCAR_{138-142}$ immobilized on magnetic beads, (ii) lack of binding of the 120–140 kDa protein to either magnetic beads alone or $rCAR_{138-142}$ immobilized on magnetic beads, and (iii) immunoprecipitation of the $CAR_{138-142}$-bound protein by an anti-vinculin mAb but not by anti-β-integrin subunit nor $p125^{FAK}$ mAbs. Based on its binding to vinculin, $CAR_{138-142}$ appears to interact with the actin cytoskeleton and thus modulates integrin binding to type IV collagen by affecting "inside-out" signal transduction.

The behaviors of $CAR_{138-142}$ and $pCAR_{138-142}$ were different, with $pCAR_{138-142}$ providing greater inhibition at similar concentrations. While not essential, Tyr phosphorylation enhances the function of the *C*-terminal region of CAR. The result is not surprising, since Tyr phosphorylation frequently increases the affinity of a signaling molecule for its ligand. The $CAR_{138-142}$ region has been proposed to be a Tyr kinase recognition site,[18] based on the Arg-X-X-Glu-X-X-Tyr motif.[39] It is possible that intracellular kinases can phosphorylate $CAR_{138-142}$ allowing for cellular modulation of activity.

Our results suggest a therapeutic potential for biomolecules whose design is based upon CAR structure. The intracellular incorporation of $pCAR_{138-142}$ and $pCAR_{138-142}$ decreases levels of melanoma cell adhesion to type IV collagen. The $pCAR_{138-142}$ peptide could be stabilized for *in vivo* use by replacement of $Tyr(PO_3H_2)$ with nonhydrolyzable analogs.[40] One could then use *in vivo* approaches such as melanoma cell receptor specific vesicles[41] to deliver CAR analogs, resulting in phenotypic alterations of cells and subsequent inhibition of binding to the ECM.

Experimental Section

Materials. All standard peptide synthesis chemicals were analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher (Pittsburgh, PA). Fmoc-4-[[(2′,4′-dimethoxyphenyl)amino]methyl]phenoxy resin (sub. level = 0.52 mmol/g) and hydroxybenotriazole (HOBt) were from Novabiochem (La Jolla, CA), and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was from Richelieu Biotechnologies (St.-Hyacinthe, Quebec). 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives [including Fmoc-$Tyr(PO_3H_2)$-OH] were obtained from Novabiochem, PerSeptive Biosystems (Framingham, MA) or Advanced Chemtech (Louisville, KY). Amino acids are of the L-configuration. Fluorescein was obtained from Sigma (St. Louis, MO). Intact type IV collagen was isolated from mouse Engelbreth–Holm–Swarm tumor as described.[42,43] Monoclonal antibody (mAb) P5D2 was prepared against the β_1-integrin subunit using methods described previously.[44] MAbs prepared against the integrin subunit α_2 (A2-IIE10) and $pp125^{FAK}$ were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The vinculin mAb was purchased from Chemicon (Temecula, CA).

The TransPort Transient Cell Permeabilization Kit was purchased from GibcoBRL (Gaithersburg, MD). The TransPort kit is composed of (i) an isotonic high-potassium HEPES intracellular buffer, (ii) a water soluble lipid derivative which initiates cell permeabilization, (iii) a protein solution which adsorbs the lipid derivative, resulting in reversal of permeabilization, and (iv) trypan blue.

P ptide Synthesis. Four peptides have been synthesized for this study. The first contains the CAR 138–142 sequence, nonphosphorylated (Val-Glu-Ile-Leu-Tyr-NH_2, designated $CAR_{138-142}$). The second contains the CAR 138–142 sequence, phosphorylated at Tyr_{142} [Val-Glu-Ile-Leu-$Tyr(PO_3H_2)$-NH_2, designated $pCAR_{138-142}$]. The third contains the CAR 138–142 sequence in reverse order, nonphosphorylated (Tyr-Leu-Ile-Glu-Val-NH_2, designated $rCAR_{138-142}$). The fourth contains

the fluorophore fluorescein on the *N*-terminus of the CAR 138–142 sequence, nonphosphorylated (designated fCAR$_{138-142}$).

Incorporation of individual amino acids was by Fmoc solid-phase methodology on an Applied Biosystems 431A peptide synthesizer using cycles described previously.[45,46] For the phosphorylated peptide pCAR$_{138-142}$, 1 equiv of Fmoc-Tyr-(PO_3H_2)-OH was used for the first coupling, followed by a second coupling with 4 equiv of Fmoc-Tyr(*t*-Bu)-OH. Peptides were cleaved from the resin and side-chain deprotected by treatment with water–trifluoroacetic acid (TFA) (1:19).[47] Fluorescein labeling of the resin-bound, side-chain-protected CAR$_{138-142}$ peptide was performed manually. A 4-fold excess of HOBt, HBTU, and fluorescein was dissolved in *N,N*-dimethylformamide (DMF) and added to the peptide-resin. An 8-fold excess of *N,N*-diisopropylethylamine was added, and the peptide-resin was placed on a bidirectional mixer for 4 h. This process was repeated until a negative ninhydrin test[48] was achieved (two times total). The peptide-resin was washed with DMF and dichloromethane and allowed to dry. The peptide was cleaved from the resin and deprotected with water–TFA (1:19) for 1 h.

Peptide Purification and Characterization. Edman degradation sequence analysis was performed on an Applied Biosystems 477A protein sequencer/120A analyzer. Peptide-resins were sequenced using "embedded" methodology[49] to ensure proper composition. Crude peptides were dissolved in trimethylamine–acetonitrile–water (1:10:40) and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Rainin AutoPrep system with a Vydac 218TP152022 C_{18} column (15–20 μm particle size, 300 Å pore size, 250 × 22 mm) at a flow rate of 5.0 mL/min. The elution gradient was 15–100% B in 85 min, where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile. Detection was at 229 nm. Analytical RP-HPLC was performed on a Hewlett-Packard 1090 liquid chromatograph equipped with a Hypersil C_{18} column (5 μm particle size, 120 Å pore size, 200 × 2.1 mm). The elution gradient was 0–100% B in 30 min at a flow rate of 0.3 mL/min. Diode array detection was at 220, 254, and 280 nm.

Fast atom bombardment mass spectroscopy (FABMS) was performed on a VG 7070E-HF instrument with a glycerol matrix. FABMS analysis of purified peptides gave the following protonated mass ($[M + H]^+$) results: CAR$_{138-142}$, $[M + H]^+$ = 635.4 Da (theoretical 635.8 Da); pCAR$_{138-142}$, $[M + H]^+$ = 715.4 Da (theoretical 715.8 Da); rCAR$_{138-142}$, $[M + H]^+$ = 635.4 Da (theoretical 635.8 Da). Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Hewlett-Packard G2025A LD-TOF with a sinapinic acid matrix. MALDI-TOF-MS analysis of fCAR$_{138-142}$ (not purified) gave $[M - H]^+$ = 951.0 Da (theoretical 950.1 Da).

Cells. M14 clone 5 human melanoma cells were propagated as described previously.[25,26] Briefly, melanoma cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate, 0.1 mg/mL gentamicin (Boehringer Mannheim, Indianapolis, IN), 50 units/mL penicillin, and 0.05 mg/mL streptomycin. Cells were passaged eight times and then replaced from frozen stocks of early passage cells to minimize phenotypic drift. All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. All media reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Permeabilization/Adhesion. Type IV collagen isolated following procedures described previously[42,43] was diluted to a concentration of 10 μg/mL in PBS and adsorbed directly onto 96-well polystyrene Immulon 1 plates (Dynatech Laboratories Inc., Chantilly, VA) overnight at 4 °C. Nonspecific binding sites were blocked with 2 mg/mL ovalbumin in PBS for 2 h at 37 °C.

Melanoma cell adhesion assays were performed as described previously[25] with minor alterations. Cells were radiolabeled overnight with 175 μCi of Trans ^{35}S-Label (>1000 Ci/mmol specific activity; ICN, Costa Mesa, CA). Cells were released from tissue culture flasks with 140 mM NaCl, 5 mM KCl, 5.5 mM D-glucose, 7 mM $NaHCO_3$, 0.05% trypsin, 0.6 mM EDTA, and 0.00045% phenol red at 37 °C, then washed twice, and resuspended in intracellular buffer (ICB) (120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 1 μM $MgCl_2$, 0.2 mM EGTA). Peptide dissolved in ICB was added to a final concentration of 1–250 μM, and the cells were permeabilized using TransPort reagents (Gibco BRL, Gaithersburg, MD). Cells were incubated at 37 °C for 10 min with the water soluble lipid derivative, the derivative was quenched with the protein solution, and 1.3 mL of adhesion media (EMEM containing 20 mM HEPES and 2 mg/mL ovalbumin) was added at 37 °C.

Initial permeabilization experiments were monitored by trypan blue exclusion. Trypan blue was added along with the lipid derivative, and the percentage of cell permeabilization was calculated by dividing the number of trypan blue positive cells by the total number of cells and multiplying by 100. Once the lipid derivative was quenched, the cells were plated and monitored for trypan blue extrusion, attachment, and spreading.

For subsequent permeabilization/adhesion experiments, adhesion media were added to the cells and they were incubated at 37 °C for 60 min ("recovery period"). The cells were then transferred to a 96-well plate (Microlite, Dynatech, Chantilly, VA) at a density of 50 000 cells/mL in a total volume of 100 μL and incubated at 37 °C for 120 min. Wells were washed three times with adhesion media. Scintillation fluid (MicroScint, Packard Chemicals, Meriden, CT) was added to the remaining adherent cells, and the plate was placed on a Beckman LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA) for quantitation of adherent cells. Adhesion percentages were based on total counts of radioactivity added to each well. Nonpermeabilized cells were treated identically, except that the TransPort reagent step was omitted.

Competition of melanoma cell adhesion assays were performed as described previously[25] using 10 μg/mL type IV collagen as substrate. Cells were incubated for the last 15 min of the "recovery period" at 37 °C with 5 μg of either an anti-α_2- or -β_1-integrin subunit mAb; then the cells, in the continued presence of the mAb, were added to the wells and allowed to adhere for 120 min at 37 °C.

Fluorescence Microscopy. Fluorescence was detected using a Zeiss epifluorescence universal microscope, 160× magnification, $\lambda_{excitation}$ = 450 nm, $\lambda_{emission}$ = 535 nm. Cells were either permeabilized or not permeabilized, treated with 100 μM peptide plus 0.003% trypan blue as described above at 37 °C, and then pipetted directly onto glass slides. Kodak Gold 400 film was used for photographs.

Affinity Isolation. Tosyl-activated magnetic beads (M-450, Dynal, Oslo, Norway) were washed with PBS and incubated for 24 h with either pCAR$_{138-142}$ or rCAR$_{138-142}$ dissolved in PBS. Fresh peptide solution was added, and the incubation step was repeated. Unoccupied binding sites were blocked with tris by incubating the beads in 1 M tris-HCl for 4 h. Beads were washed and stored in PBS at 4 °C until use. In addition, tris-blocked magnetic beads were prepared without peptide. The Tris-blocked beads were used to reduce nonspecific binding of proteins to either pCAR$_{138-142}$ or rCAR$_{138-142}$-coated beads.

Cells were harvested at 80–90% confluence, washed with PBS, and lysed with lysis buffer (0.25% Triton X-100, 75 mM NaCl, 500 μM sodium *o*-vanadate, 25 mM Tris-HCl, 2.5 mM EDTA, 5 μg/mL aprotinin, 5 μg/mL leupeptin) at 4 °C. The cell lysates were precleared with the Tris-blocked magnetic beads by constant mixing for 1 h at 4 °C to remove any proteins that could bind nonspecifically to the magnetic beads. The beads and nonspecifically bound proteins were magnetically pelleted, and the precleared lysates were incubated with the peptide-coated magnetic beads with constant mixing for 2 h at 4 °C. The beads were again pelleted using a magnet and washed three times with PBS. A buffer containing 0.1 M Tris-HCl, 1 mM EDTA, and 6 M guanidine hydrochloride (pH 8.5) was added to the beads, and they were allowed to incubate at room temperature for 1 h with constant mixing. The proteins were separated from the beads using a Sep-Pak C_{18} cartridge from Waters (Milford, MA). This additional step allowed the magnetic beads to be completely removed from the sample since their presence was found to interfere with the subsequent

procedures. The column was first washed with water to remove salts and then washed with 80–100% aceonitrile to elute the proteins. The eluate was lyophilized and dissolved in lysis buffer.

Immunoprecipitation. Goat antimouse Sepharose beads (Zymed, San Francisco, CA) were incubated with the appropriate monoclonal antibody for at least 4 h at 4 °C with mixing. The beads were washed with lysis buffer (0.25% Triton X-100, 75 mM NaCl, 25 mM Tris-HCl, 0.5 mM EDTA, 5 μg/mL aprotinin, 5 μg/mL leupeptin). Cell lysate was added to the beads, and they were incubated at 4 °C for at least 4 h with mixing. The lysate was removed, and the beads were washed three times with lysis buffer.

Western Blotting. Laemmli buffer[50] was added to the samples, and they were heated for 5 min at 100 °C. Sample fractions were electrophoresed by 10% SDS–PAGE (Bio-Rad, Hercules, CA) and transferred to nitrocellulose (Micron Separations, Inc., Westboro, MA). The nitrocellulose was washed with 0.1 M $NaHCO_3$ for 5 min and incubated in 1 mg of sulfosuccinimidobiotin (Pierce, Rockford, IL) in 0.1 M $NaHCO_3$ for 1 h. Nonspecific binding sites were blocked with TBST plus 2% BSA for 1 h. The membrane was incubated with 20 μg of ImmunoPure streptavidin horseradish peroxidase (Pierce) in TBST plus 2% BSA for 1 h by three washes of TBST for 15 min each. Enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) were used for detection of biotinylated proteins. Molecular weight standards (Sigma) were β-galactosidase (120 kDa), bovine serum albumin (87 kDa), and ovalbumin (48.1 kDa).

Acknowledgment. This work was supported by the National Institutes of Health (KD 44494, AR 01929, and CA 63671 to G.B F.; CA 21463, CA 29995, and EY 09065 to L.T.F.) and the American Cancer Society. L.T.F. is an Allen-Pardee Professor.

References

(1) Weitzman, J. B.; Pujades, C.; Hemler, M. E. Integrin α Chain Cytoplasmic Tails Regulate "Antibody-redirected" Cell Adheison, Independently of Ligand Binding. *Eur. J. Immunol.* **1997**, *27*, 78-84.
(2) Ginsberg, M. H.; Du, X.; Plow, E. F. Inside-out Integrin Signalling. *Curr. Opin. Cell. Biol.* **1992**, *4*, 766–770.
(3) Dedhar, S.; Saulnier, R. Alterations in Integrin Receptor Expression in Chemically Transformed Human Cells: Specific Enhancement of Laminin and Collagen Receptor Complexes. *J. Cell Biol.* **1990**, *110*, 481–489.
(4) Santala, P.; Larjava, H.; Nissinen, L.; Riikonen, T.; Maatta, A.; Heino, J. Suppressed Collagen Gene Expression and Induction of Alpha 2 Beta 1 Integrin-type Collagen Receptor in Tumorigenic Derivatives of Human Osteogenic Sarcoma (HOS) Cell Line. *J. Biol. Chem.* **1994**, *269*, 1276–1283.
(5) Vihinen, P.; Riikonen, T.; Laine, A.; Heino, J. Integrin α2β1 in Tumorigenic Human Osteosarcoma Cell Lines Regulates Cell Adhesion, Migration, and Invasion by Interaction with Type I Collagen. *Cell Growth Different.* **1996**, *7*, 439–447.
(6) Klein, C. E.; Dressel, D.; Steinmayer, T.; Mauch, C.; Eckdes, B.; Kreig, T.; Bankert, R. B.; Weber, L. Integrin Alpha 2 Beta 1 is Upregulated in Fibroblasts and Highly Aggressive Melanoma Cell in Three-Dimensional Collagen Lattices and Mediates the Reorganization of Type I Collagen Fibrils. *J. Cell Biol.* **1991**, *115*, 1427–1436.
(7) Danen, E. H. J.; van Muijen, G. N. P.; van de Wiel-van Kemenade, E.; Jansen, K. F. J.; Ruiter, D. J.; Figdor, C. G. Regulation of Integrin-mediated Adhesion to Laminin and Collagen in Human Melanocytes and in Non-metastatic and Highly Metastatic Human Melanoma Cells. *Int. J. Cancer* **1993**, *54*, 315–321.
(8) Kuhn, K; Eble, J. The Structural Basis of Integrin-ligand Interactions. *Trends Cell Biol.* **1994**, *4*, 256–261.
(9) Kern, A.; Eble, J.; Golbik, R.; Kuhn, K. Interaction of Type IV Collagen with the Isolated Integrins α1β1 and α2β1. *Eur. J. Biochem.* **1993**, *215*, 151–159.
(10) Santoro, S. A.; Zutter, M. M. The alpha 2 beta 1 integrin: A Collagen Receptor on Platelets and Other Cells. *Thromb. Haemostasis* **1995**, *74*, 813–821.
(11) Etoh, T.; Thomas, L.; Pastel-Levy, C.; Colvin, R. B.; Mihm, M. C., Jr.; Byers, H. R. Role of Integrin α2β1 (VLA-2) in the Migration of Human Melanoma Cells on Laminin and Type IV Collagen. *J. Invest. Dermatol.* **1993**, *100*, 640–647.
(12) Morelli, J. G.; Yohn, J. J.; Zekman, T.; Norris, D. A. Melanocyte Movement *In Vitro*: Role of Matrix Proteins and Integrin Receptors. *J. Invest. Dermatol.* **1993**, *101*, 605–608.
(13) Yoshinaga, I. G.; Vink, J.; Dekker, S. K.; Mihm, M. C. J.; Byers, H. R. Differential Effect of Magnesium and Valcium on Integrin-mediated Melanoma Cell Migration on Type IV Collagen and Fibronectin. *Melanoma Res.* **1993**, *3*, 435–441.
(14) Vink, J.; Dekker, S. K.; Van Leeuwen, R. L.; Vermeer, B. J.; Bruijn, J. A.; Byers, H. R. Role of Beta 1 Integrins in Cell Spreading and Migration of Human Nevomelanocytes and Dysplastic Nevi Cells on Collagen Type IV and Laminin. *Pigment Cell Res.* **1994**, *7*, 339–347.
(15) Knutson, J. R.; Iida, J.; Fields, G. B.; McCarthy, J. B. CD45/Chrondroitin Sulfate Proteoglycan and α2β1 Integrin Mediate Human Melanoma Cell Migration on Type IV Collagen and Invasion of Basement Membranes. *Mol. Biol. Cell* **1996**, *7*, 383–396.
(16) Langholz, O.; Rockel, D.; Mauch, C.; Kozlowska, E.; Bank, I.; Krieg, T.; Eckes, B. Collagen and Collagenase Gene Expression in Three-dimensional Collagen Lattices are Differentially Regulated by Alpha 1 Beta 1 and Alpha 2 Beta 1 Integrins. *J. Cell Biol.* **1995**, *131*, 1903–1915.
(17) Riikonen, T.; Westermarck, J.; Koivisto, L.; Broberg, A.; Kahari, V. M.; Heino, J. Transforming Growth Factor-beta Regulates Collagen Contraction by Increasing Alpha 2 Beta 1 Integrin Expression in Osteogenic Cells. *J. Biol. Chem.* **1995**, *270*, 13548–13552.
(18) Pullman, W. E.; Bodmer, W. F. Cloning and Characterization of a Gene that Regulates Cell Adhesion. *Nature* **1992**, *356*, 529–532.
(19) Yamamoto, H.; Itoh, F.; Hinoda, Y.; Imai, K. Inverse Association of Cell Adhesion Regulator Messenger RNA expression with Metastasis in Human Colorectal Cancer. *Cancer Res.* **1996**, *56*, 3605–3609.
(20) Koyama, K.; Emi, M.; Nakamura, Y. The Cell Adhesion Regulator (CAR) Gene, TaqI and Insertion/Deletion Polymorphisms, and Regional Assignment to the Peritelomeric Region of 16q by Linkage Analysis. *Genomics* **1993**, *16*, 264–265.
(21) Lin, Y. Z.; Yao, S. Y.; Yeach, R. A.; Torgerson, T. R.; Hawiger, J. Inhibition of Nuclear Translocation of Transcription Factor NF-kB by a Synthetic Peptide Containing a Cell Membrane-permeable Motif and Nuclear Localization Sequence. *J. Biol. Chem.* **1995**, *270*, 14255–14258.
(22) Wange, R. L.; Isakov, N.; Burke, T. R.; Otaka, A.; Roller, P. P.; Watts, J. D.; Aebersold, R.; Samelson, L. E. $F_2(Pmp)_2$-TAMζ$_3$, a Novel Competetive Inhibitor of the Binding of ZAP-70 to the T Cell Antigen Receptor, Blocks Early T Cell Signaling. *J. Biol. Chem.* **1995**, *270*, 944–948.
(23) Damaj, B. B.; McColl, S. R.; Mahana, W.; Crouch, M. F.; Naccache, P. H. Physical Association of $G_{i2}\alpha$ with Interleukin-8 Receptors. *J. Biol. Chem.* **1996**, *271*, 12783–12789.
(24) Gibco-BRL Life Technologies. Product literature, 1994.
(25) Miles, A. J.; Skubitz, A. P. N.; Furcht, L. T.; Fields, G. B. Promotion of Cell Adhesion by Single-stranded and Triple-helical Peptide Models of Basement Membrane Collagen α1(IV)531–543: Evidence for Conformationally Dependent and Conformationally Independent Type IV Collagen Cell Adhesion Sites. *J. Biol. Chem.* **1994**, *269*, 30939–30945.
(26) Miles, A. J.; Knutson, J. R.; Skubitz, A. P. N.; Furcht, L. T.; McCarthy, J. B.; Fields, G. B. A Peptide Model of Basement Membrane Collagen α1(IV)531–543 Binds the α3β1 Integrin. *J. Biol. Chem.* **1995**, *270*, 29047–29050.
(27) Wilke, M. S.; Furcht, L. T.; Human Keratinocytes Adhere to a Unique Heparin-Binding Peptide Sequence Within the Triple Helical Region of Type IV Collagen. *J. Invest. Dermatol.* **1990**, *95*, 264–270.
(28) Vostal, J. G.; Shulman, N. R. Vinculin is a Major Platelet Protein that Undergoes Ca^{2+}-dependent Tyrosine Phosphorylation. *Biochem. J.* **1993**, *294*, 675–680.
(29) Fennell, D. F.; Whatley, R. E.; McIntyre, T. M.; Prescott, S. M.; Zimmerman, G. A. Endothelial Cells Reestablish Functional Integrity after Reversible Permeabilization. *Arteriosclerosis Thromb.* **1991**, *11*, 97–106.
(30) Barja, P.; Alavi-Nassab, A.; Turck, C. W.; Freire-Moar, J. Inhibition of T Cell Activation by Protein Kinase C Pseudosubstrates. *Cell. Immunol.* **1994**, *153*, 28–38.
(31) Schieffer, B.; Paxton, W. G.; Chai, Q.; Marrero, M. B.; Bernstein, K. E. Angiotensin II Control $p21^{ras}$ Activity Via $pp60^{c-src}$. *J. Biol. Chem.* **1996**, *271*, 10329–10333.
(32) Bodor, N.; Prokai, L.; Wu, W. M.; Harag, H.; Jonalagadda, S.; Kawamura, M.; Simpkins, J. A Strategy for Delivering Peptides into the Central Nervous System by Sequential Metabolism. *Science* **1992**, *257*, 1698–1700.
(33) Sonnenberg, A. Integrins and Their Ligands. *Curr. Top. Microbiol. Immunol.* **1993**, *184*, 7–35.
(34) Zachary, I.; Rozengurt, E. Focal Adhesion Kinase ($p125^{FAK}$): A Point of Convergence in the Action of Neuropeptides, Integrins, and Oncogenes. *Cell* **1992**, *71*, 891–894.
(35) Harte, M. T.; Hildebrand, J. D.; Burnham, M. R.; Bouton, A. H.; Parsons, J. T. $p130^{Cas}$, a Substrate Associated with v-src and v-crk, Localizes to Focal Adhesions and Binds to Focal Adhesion Kinase. *J. Biol. Chem.* **1996**, *271*, 13649–13655.

(36) Reid, D. M.; Jones, C. E.; Luo, C. Y.; Shulman, N. R.; Immunoglobulins from Normal Sera Bind Platelet Vinculin and Talin and Their Proteolytic Fragments. *Blood* **1993**, *81*, 745–751.
(37) Sung, K. L.; Yang, L.; Whittemore, D. E.; Shi, Y.; Jin, G.; Hsieh, A. H.; Akeson, W. H.; Sung, L. A. The Differential Adhesion Forces of Anterior Cruciate and Medial Collateral Ligament Fibroblasts: Effects of Tropomodulin, Talin, Vinculin, and Alpha-actinin. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9182–9187.
(38) Stevens, G. R.; Zhang, C.; Berg, M. M.; Lambert, M. P.; Barber, K.; Cantallops, I.; Routtenberg, A.; Klein, W. L. CNS Neuronal Focal Adhesion Kinase Forms Clusters that Co-localize with Vinculin. *J. Neurosci. Res.* **1996**, *46*, 445-455.
(39) Patschinsky, T.; Hunter, T.; Esch, F. S.; Cooper, J. A.; Setton, J. M. Analysis of the Sequence of Amino Acids Surrounding Sites of Tyrosine Phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 973–977.
(40) Lauer, J. L.; Fields, G. B.; Design and use of Synthetic Peptides As Biological Models. In *Protein Structure*; Angeletti, R. H., Ed.; Academic Press: Orlando, FL, 1997; in press.
(41) Yu, Y. C.; Berndt, P.; Tirrell, M.; Fields, G. B. Self-Assembling Amphiphiles for Construction of Protein Molecular Architecture. *J. Am. Chem. Soc.* **1996**, *118*, 12515–12520.
(42) Tsilibary, E. C.; Charonis, A. S. The Role of the Main Non-collagenous Domain (NC1) in Type IV Collagen Self-assembly. *J. Cell Biol.* **1986**, *103*, 2467–2473.
(43) Koliakos, G. G.; Kouzi-Koliakos, K.; Furcht, L. T.; Reger, L. A.; Tsilibary, E. C. The Binding of Heparin to Type IV Collagen: Domain Specificity with Identification of Peptide Sequences from the α1(IV) and α2(IV) Which Preferentially Bind Heparin. *J. Biol. Chem.* **1989**, *264*, 2313–2323.
(44) Wayner, E. A.; Carter, W. G. Identification of Multiple Cell Adhesion Receptors for Collagen and Fibronectin in Human Fibrosarcoma Cells Possessing Unique Alpha and Common Beta Subunits. *J. Cell Biol.* **1987**, *105*, 1873–1884.
(45) Fields, C. G.; Loffet, A.; Kates, S. A.; Fields, G. B. The Development of High-performance Liquid Chromatographic Analysis of Allyl and Allyloxycarbonyl Side-chain-protected Phenylthiohydantoin Amino Acids. *Anal. Biochem.* **1992**, *203*, 245–251.
(46) Fields, C. G.; Lloyd, D. H.; Macdonald, R. L.; Otteson, K. M.; Noble, R. L. HBTU Activation for Automated Fmoc Solid-phase Peptide Synthesis. *Pept. Res.* **1991**, *4*, 95–101.
(47) Fields, C. G.; Fields, G. B. Minimization of Tryptophan Alkylation Following 9-Fluorenylmethoxycarbonyl Solid-phase Peptide Synthesis. *Tetrahedron Lett.* **1993**, *34*, 6661–6664.
(48) Fields, G. B.; Tian, Z.; Barany, G. Principles and Practice of Solid-phase Peptide Synthesis. *Synthetic Peptides: A User's Guide*; W.H. Freeman & Co.: New York, 1993; pp 77–183.
(49) Fields, C. G.; VanDrisse, V. L.; Fields, G. B. Edman Degradation Sequence analysis of Resin-bound Peptides Synthesized by 9-Fluorenylmethoxycarbonyl Chemistry. *Pept. Res.* **1993**, *6*, 39–47.
(50) Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

JM970206J